Assay of Intracellular Free Calcium in Suspended B Cells (with Fluoroskan Ascent)

AfCS Procedure Protocol ID PP0000001100
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This protocol describes a method to assess concentrations of free cytoplasmic calcium (Ca^{2+}) in mouse splenic B cells in the absence and presence of ligands for cell surface receptors. This objective is accomplished with the Ca^{2+}-sensitive fluorescent dye, Fluo-3, which permeates cells as an ester and is hydrolyzed in the cell to its Ca^{2+}-sensitive acidic form. Fluorescence is measured over time with suspended cells that have been washed free of extracellular dye. Free [Ca^{2+}] is estimated as described below (see *Calculations*).

A Fluoroskan Ascent Microplate Fluorometer and Ascent software (Thermo-Labsystems) are used to measure fluorescence and for initial analysis of results. For detailed time courses, internal dispensers are loaded with solutions of ligands and used to add the ligand to specified wells. After additions, the contents of wells are mixed in the fluorometer by rapid rotation of the plate holder (shake program settings: diameter of the shake is 5 mm; speed is 240 rotations per min). Multiple wells are read in sequence at set intervals.

Treatment of Cells and Detection of Intracellular Calcium with Fluo-3

1. Determine the number of cells required. This equals the number of samples x 1.5 x 10^6 cells/sample. Cells are loaded with dye in aliquots of 3 ml containing 15 x 10^6 cells. Determine the number of aliquots (3 ml; 15 x 10^6 cells) required.

No. of samples required	No. of cells needed	No. of 3-ml aliquots (wells) to be used for loading
1-10	15 x 10^6	1
11-20	30 x 10^6	2
21-30	45 x 10^6	3
31-40	60 x 10^6	4

2. Mix equal volumes (15 µl/3-ml aliquot) of 1 mM Fluo-3, AM and 20% Pluronic F-127.
3. Suspend the desired number of freshly isolated splenic B cells at 5 x 10^6 cells/ml in Supplemented Iscove's Modified Dulbecco's Medium (SIMDM) and distribute 3 ml into individual wells of a 6-well (growth area 9.5 cm^2) ultra-low attachment plate.
4. Add 24 µl of the Fluo3 mixture (step 2) containing 0.5 mM Fluo-3, AM (4 µM final concentration) and 10% Pluronic F-127 to each well containing cells.
5. Mix gently by rocking the plate five times.
6. Incubate at 37 °C in air with 5% CO_2 for 30 min; mix every 10 min.
7. Transfer the cells (up to 4 wells) to a 15-ml conical polypropylene tube. Use an additional 1 ml of SIMDM to rinse the culture wells and combine with the pooled cells. Centrifuge at 400 x g for 5 min at room temperature.

8. Remove the medium and suspend the cells to the original volume (step 3) of fresh SIMDM; return the suspended cells (3 ml/well) to unused wells of a 6-well ultra-low attachment plate.
9. Incubate at 37 °C in air with 5% CO_2 for 30 min; mix every 10 min.
10. Transfer the cells to a new 15-ml conical tube, rinse the wells with 1 ml of SIMDM, and centrifuge at 400 x g for 5 min at room temperature.
11. Wash the cells once with 15 ml of Hanks' Balanced Salt Solution-Bovine Serum Albumin (HBSS-BSA) at room temperature and collect as above.
12. Suspend the cells in HBSS-BSA (0.5 ml for each aliquot/well of cells). Determine the cell density, and adjust the cell density to 8.3×10^6 cell/ml as necessary with HBSS-BSA (about 1 ml total/aliquot). Cells can be stored at room temperature for up to 2 hr in the dark. Storage in darkness avoids prolonged stimulation of the fluorescent dye by ambient light.
13. Distribute 0.090 ml of the cell suspension into appropriate wells of a 96-well black-walled plate and incubate in a 37 °C environmental chamber for 5 min. Note: temperature equilibration can also be done in the fluorometer chamber for initial samples.
14. Transfer the plate to the fluorometer and measure baseline fluorescence for 10 min (this also serves to equilibrate the cells in the fluorescence chamber). The fluorescence of Fluo-3 is measured with filters for excitation at 485 nm and for emission at 538 nm.
15. At the desired time (usually after 10 min, but when baseline fluorescence, e.g., resting Ca^{2+} and temperature, are stable), add 0.010 ml of ligand (10X final concentration in HBSS-BSA) or vehicle (in HBSS-BSA) to appropriate wells to begin treatments. (Note: vehicle controls constitute matching dilutions of solvents in which ligands are dissolved and stored.) Mix for 1 sec and measure fluorescence (F) for 10 min. (Note: for detailed time courses, ligands are added rapidly with internal dispensers; less detailed measurements are made with manual additions and contain delays of up to 20 sec between addition of ligand and measurement of fluorescence.)
16. After 10 min of exposure to ligand, add 100 µl of 1% NP-40 to each well to release dye from the cells. Mix for 10 sec and wait for 2 min; then measure fluorescence (F_{max} = total fluorescence of dye at saturating Ca^{2+}) for 10 measurements at 10 sec intervals. The fluorescence should be stable.
17. Add 20 µl of 0.5 M EGTA to each well to chelate Ca^{2+}, mix for 10 sec, and wait for 2 min; then measure fluorescence (F_{min} = total fluorescence of dye in the absence of free Ca^{2+}) for 10 measurements at 10 sec intervals. The fluorescence should be stable.

Calculations

$$[\text{free } Ca^{2+}] = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where the K_d for Fluo-3 is assumed = 390 nM

Reagents and Materials

Fluo-3, acetoxymethyl (AM), 1 mM solution in dimethylsulfoxide (DMSO): Molecular Probes; catalog no. F-14218

Pluronic F-127, 20% solution in dimethylsulfoxide (DMSO): Molecular Probes; catalog no. P-3000

Supplemented Iscove's Modified Dulbecco's Medium (SIMDM): AfCS Solution Protocol ID PS0000005600

6-well ultra-low attachment plates: Corning/Costar; catalog no. 07-200-601

Conical polypropylene tubes, 15 ml: Greiner; catalog no. 188261

Hanks' Balanced Salt Solution-Bovine Serum Albumin (HBSS-BSA), pH 7.4: AfCS Solution Protocol ID PS0000003200

96-well black-walled plate: Thermo-Labsystems; catalog no. 9502867

Fluoroskan Ascent Microplate Fluorometer: Thermo-Labsystems; catalog no. 5210470

Fluorometer dispensers: Thermo-Labsystems; catalog no. 2805630

Ascent software: Thermo-Labsystems; catalog no. 5185470CD

NP-40 (Igepal CA-630), 1%: AfCS Solution Protocol ID PS0000003900

0.5 M EGTA, pH 8.0: AfCS Solution Protocol ID PS0000002700

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